An Inactivating Mutation within the First Extracellular Loop of the Thyrotropin Receptor Impedes Normal Posttranslational Maturation of the Extracellular Domain

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The TSH receptor (TSHR), a member of the large family of G protein-coupled receptors, controls both function and growth of thyroid cells; hence, mutations of this receptor result in thyroid dysfunction. Here, we took advantage of the description of a new inactivating *TSHR* mutation (Q489H) in two brothers with hypothyroidism, to precise maturation, intracellular trafficking, exporting pathways, and activation mechanisms of this receptor. Functional characterization of the Q489H-TSHR in transiently transfected HEK293 cells showed cell surface expression, normal TSH binding affinity, and its inability to generate intracellular cAMP in response to TSH stimulation. Western blot analysis of the whole membrane proteins or proteins expressed at the cell surface showed that Q489H-TSHR expressed in HEK293 transfected cells are restricted to mannose-rich uncleaved receptor. Analysis of the export pathway toward cell surface indicated that both Q489H and wild-type receptors followed the same intracellular route to cell surface throughout endoplasmic reticulum and Golgi apparatus. This study shows that Q489H substitution impedes complete glycosylation of TSHR extracellular domain within the Golgi apparatus and that Q489H-TSHR expressed at the cell surface is unable to undergo intramolecular cleavage as well as to switch toward an active conformation under TSH stimulation. Altogether, our results show that 1) Q489H substitution within the first extracellular loop induces a misfolding of TSHR, blocking it into an inactive conformation and impeding complete glycosylation and intramolecular cleavage, and 2) a misfolded G protein-coupled receptor can bypass endoplasmic reticulum or Golgi apparatus quality control and reach the cell surface as an immature receptor. **(*Endocrinology* 150: 1043–1050, 2009)**

TSH and the TSH receptor (TSHR) are key proteins in the control of thyroid function. TSH, upon its interaction with the TSHR, controls different thyroid functions, such as iodine uptake and organification, production and release of iodothyronines from the gland, and promotion of thyroid growth. It also acts as a factor protecting thyroid cells from apoptosis and plays a critical role in ontogeny (1–3).

The TSHR along with LH/chorionic gonadotropin (CG) and FSH receptors are related members of the rhodopsin/β-adrenergic receptors family of seven-transmembrane domain G protein-coupled receptors (GPCR) superfamily and constitute the glycoprotein hormone receptors (GPHR) subfamily. TSH binding to TSHR, leads to the stimulation of second messenger pathways involving predominantly cAMP and, at high concentrations, ino-

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Abbreviations: CG, Chorionic gonadotropin; DTT, dithiothreitol; ECD, extracellular domain; EndoH, endoglycosidase H; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GPHR, glycoprotein hormone receptor; GRASP65, Golgi reassembly stacking protein 65; PNGase F, *N*-glycosidase F; Tg, thyroglobulin; TMD, transmembrane domain; TSHR, TSH receptor; WT-TSHR, wild-type TSHR.

sitol 1,4,5-trisphosphate and diacylglycerol, ultimately resulting in the modulation of thyroidal gene expression (4).

In contrast with many other members of the GPCRs, the TSHR has a large extracellular domain (ECD), accounting for about one half of the receptor molecular size and mainly consisting of leucine-rich repeats important for receptor structure and activation. Intramolecular interactions between the functionally intact ECD and the extracellular loops (ECL) of the transmembrane domain (TMD) maintain the receptor in a constrained and closed conformation stabilizing the inactive configuration of the TSHR (5). The TSHR is unique among GPHR in that expressed cell surface mature receptors are cleaved into two subunits (6, 7). The α-subunit, 56 kDa, corresponds to the N-terminal part of the ECD, and the β-subunit, 42 kDa, composes the C-terminal end of the ECD, the TMD, and the intracellular domains. The two subunits are held together by disulfide bridges, and the α-subunit may be shed upon reduction of these bonds (7, 8).

Other TSHR posttranslational maturations, such as *N*-glycosylation, sialylation, or sulfation have been described (9). The TSHR is primarily synthesized as a 95-kDa mannose-rich monomer and then a supplementary *N*-glycosylation with complex-type sugars occurs on six *N*-glycosylation sites within the ECD. It has been reported that at least four glycosylation sites must be fully glycosylated to allow normal TSH binding affinity and TSH-induced cAMP increase (10). A complete inhibition of *N*-glycosylation by tunicamycin completely impedes TSH binding on cell surface (11).

Over the past decade, loss-of-function mutations of human TSHR have been described in association with a phenotypic spectrum ranging from asymptomatic hyperthyrotropinemia to congenital hypothyroidism with severe thyroid hypoplasia or not detected thyroid tissue in normal position (apparent athyreosis) (12–22). All reported mutations decreased adenylate cyclase stimulation upon TSH stimulation through reduced transduction, affinity for TSH, or cell surface expression. Misfolding of mutant receptors leading to endoplasmic reticulum (ER) retention and degradation through the proteasome pathway has been described for several GPCR, including TSHR (23). However, alterations of posttranslational maturation beyond the exit of ER toward Golgi apparatus leading to a defect of GPCR signaling remain very rare (24).

In this study, we showed that a Q489H substitution, localized within the first ECL (ECL1) of the TSHR, completely impedes normal posttranslational glycosylation and intramolecular cleavage and blocks TSH-induced adenylate cyclase activity, but it does not reduce affinity for TSH.

Materials and Methods

Patients

The two patients were the first and third children of consanguineous North African parents. Systematic neonatal screening for congenital hypothyroidism, performed in France since 1979, revealed TSH elevation in both infants. Hypothyroidism was diagnosed within the first month of life, and therapy was initiated immediately. The neonatal course was otherwise uneventful. All thyroid metabolic and morphological analyses performed are summarized in Table 1. Informed consent was obtained to perform genetic studies. The study was reviewed and approved by the Necker Hospital Ethics Committee.

TSHR gene familial linkage analysis and sequencing

Genomic DNA was isolated from peripheral blood leukocytes of the patients and their euthyroid mother and sister. Linkage between the *TSHR* gene and the phenotype was evaluated using an intragenic microsatellite marker and an AT repeat within the second intron of the *TSHR* locus as well as two extragenic microsatellites, D14S1035 and D14S1037, located on each side of the gene as previously described (25, 26). The fragments containing exons 1–9 and the entire length of exon 10 were amplified, purified, and sequenced as described elsewhere (26).

Cell culture and transfections

Construction of the expression vector (pSG5) containing the full-length coding sequence of the wild-type TSHR (WT-TSHR) is described elsewhere (17). The Q489H mutation was introduced within the wild-type original construct by PCR as previously described (17). The resulting construct (Q489H-TSHR) was verified by sequencing both strands. The Golgi reassembly stacking protein 65 (GRASP65)-green fluorescent protein (GFP) vector was described elsewhere (27). Human embryonic kidney fibroblast cells (HEK293) were grown in DMEM supplemented with 10% total bovine serum and 1% penicillin, streptomycin, and fungizone (Life Technologies, Inc., BRL, Bethesda, MD). Cells were transiently transfected with 100, 250, 500, or 750 ng of the various vector constructions in 12-well plates using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions.

cAMP accumulation and binding assays

Intracellular cAMP accumulation was measured from HEK293 transfected cells with or without bovine TSH stimulation, and [^{125}I]TSH binding experiments were performed as previously described. Transfection of empty pSG5 vector was used as a negative control (17).

TABLE 1. Summary of thyroid metabolic data and morphological exploration of all available family members

	Plasma TSH (μU/ml)	fT_3 (pmol/liter)	fT_4 (pmol/liter)	Tg (ng/ml)	^{123}I scintigraphy	Neck MRI and US
Reference values						
Neonatal	<30	2.5–5.5	7–16	2–5		
Adult	0.3–3.6 μU/ml					
I:1 Adult	4.8	NP	14.2	NP	NP	Multinodular thyroid
II:1 Neonatal	180	0.8	1	3.6	No fixation	No thyroid tissue
II:2 Adult	5.5	NP	11.7	NP	NP	Normal
II:3 Neonatal	60	NP	2.6	5.4	No fixation	No thyroid tissue

I:1, Mother; II:1, first sibling with hypothyroidism; II:2, second sibling; II:3, third sibling with hypothyroidism; fT3, Free serum T_3; fT4, free serum T_4; MRI, magnetic resonance imaging; NP, not performed; US, ultrasound.

Fluorescence-activated cell sorting (FACS) and confocal analysis

Flow immunocytometry experiments (FACS) were performed as previously described with nonpermeabilized HEK293 transfected cells (12) using the T5–317 antibody that recognizes a region of the ECD (7). Fluorescent immunocytochemistry was performed using the T5–34 anti-TSHR antibody directed against the putative deleted fragment of the ECD of the TSHR α-subunit in nonpermeabilized cells (28), whereas the anti-calnexin and anti-TGN46 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used with Triton- or digitonin-permeabilized cells, respectively. The GRASP65-GFP was visualized directly after transfection. Analysis by confocal microscopy was performed as previously described (17).

Western blot analysis

Migration and blotting of total cellular membrane protein extracts from transfected cells under reducing conditions were carried out as previously described (29) using the T5–317 or T5–34 antibodies against the ECD of the TSHR α-subunit and the T3-365 antibody directed against the intracellular domain of the TSHR β-subunit (7). To explore the glycosylation status of the different forms of the TSHR, 40 μg Triton-solubilized protein from crude membranes were deglycosylated by endoglycosidase H (EndoH) and *N*-glycosidase F (PNGase F) (New England Biolabs, Inc., Beverly, MA) according to the manufacturer's instructions.

Cell surface biotinylation

Cell surface proteins of nonpermeabilized transfected HEK293 cells expressing WT-TSHR or Q489H-TSHR were biotinylated with a thiol-cleavable amine-reactive biotinylation reagent as described by the manufacturer (Pierce, Rockford, IL). Briefly, 48 h after transient transfection of HEK293 cells with a plasmid expressing WT-TSHR or Q489H-TSHR, cells were washed twice with PBS at 4 C and incubated with the cross-linker in PBS for 30 min at 4 C. Quenching solution was then added for 2 min at 4 C, cells were scraped, and they were washed twice in Tris-buffered saline. After the last centrifugation, cell pellets were sonicated in the lysis buffer and incubated for 30 min at 4 C. The supernatant was added to Neutravidin gel prepared as recommended by the manufacturer. After extended washing with the wash buffer, biotinylated protein was eluted from the gel in SDS-PAGE buffer containing dithiothreitol (DTT) (50 mM) for 60 min at room temperature. Western blot was performed on isolated biotinylated proteins using T5–34 antibody against ECD of TSHR.

Shedding of the ECD from transfected HEK293 cell surface

The ECD was shed from the cell surface by DTT treatment as previously described (29). Western blot was performed with T5–34 antibody on aliquots of concentrated supernatant and solubilized cell membranes.

Results

Identification of the Q489H mutation in the TSHR gene

Linkage analysis with intragenic and extragenic markers was consistent with involvement of *TSHR* in this familial form of thyroid dysgenesis (Fig. 1A). Direct sequencing of the 10 *TSHR* exons revealed a unique homozygous C-to-G mutation at nucleotide 1467 (1467C→G) in exon 10 (Fig. 1B), leading to a glutamine-to-histidine substitution at position 489 (Q489H). This amino acid, located within the ECL1 of the TMD, is highly conserved among different species as well as in the other members of the GPHR subfamily in humans (Fig. 1C).

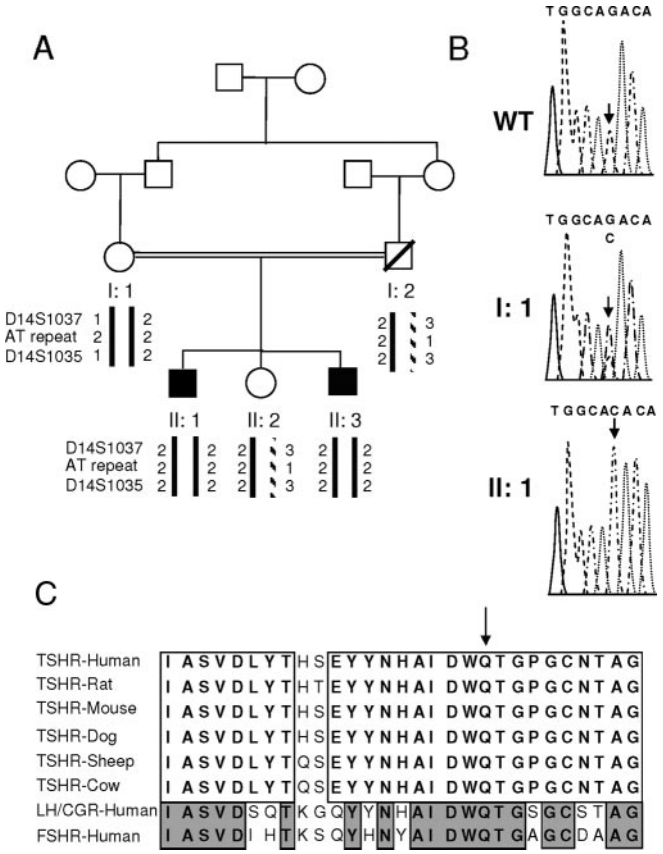

FIG. 1. Identification of the Q489H mutation in the *TSHR* gene. A, Pedigree showing the intrafamilial segregation pattern of genetic markers used for *TSHR* locus-specific analysis (*top to bottom*: D14S1035, *TSHR*at, and D14S1037). *Black symbols* represent affected patients. Microsatellites were deduced for the father. B, Genomic sequence analysis of exon 10 of human *TSHR*. Genomic sequence in a wild-type individual (WT), the mother (I:1), and a sibling with hypothyroidism (II:1); the *arrows* point to the mutation. C, Alignment of TSHR amino acid sequences from rat, mouse, dog, sheep, cow, and human and human LHCGR and FSHR. Amino acids shared by TSHR homologs are in *bold* and *inside a box*, those also shared by human LHCGR and FSHR are highlighted in *gray*, and the glutamine residue (Q) changed by the Q489H mutation is indicated by an *arrow*.

Functional characterization of the Q489H mutant receptor

In transiently transfected HEK293 cells, cAMP accumulation increased with TSH concentration in cells expressing WT-TSHR, whereas no cAMP accumulation was observed upon TSH challenging in Q489H-TSHR-expressing cells, even at high TSH concentrations (Fig. 2A). Basal cAMP accumulation increased with the amount of WT-TSHR transfected DNA (100, 250, and 750 ng DNA) (Fig. 2B). Although not significant when compared with pSG5-transfected cells, an increase of the basal activity was observed with 100 ng Q489H-TSHR-transfected DNA, which did not further increased with 250 ng transfected DNA (Fig. 2B). With 750 ng transfected Q489H-TSHR DNA, basal cAMP decreased.

Confocal analysis on nonpermeabilized cells with the T5–34 antibody and FACS analysis with the T5–317 antibody, both monoclonal antibodies directed against the ECD, showed a similar amount of cell-surface expression of the mutant receptor compared with the wild-type receptor (Fig. 3, A and B). A competition study performed with ^{125}I-labeled bovine TSH showed

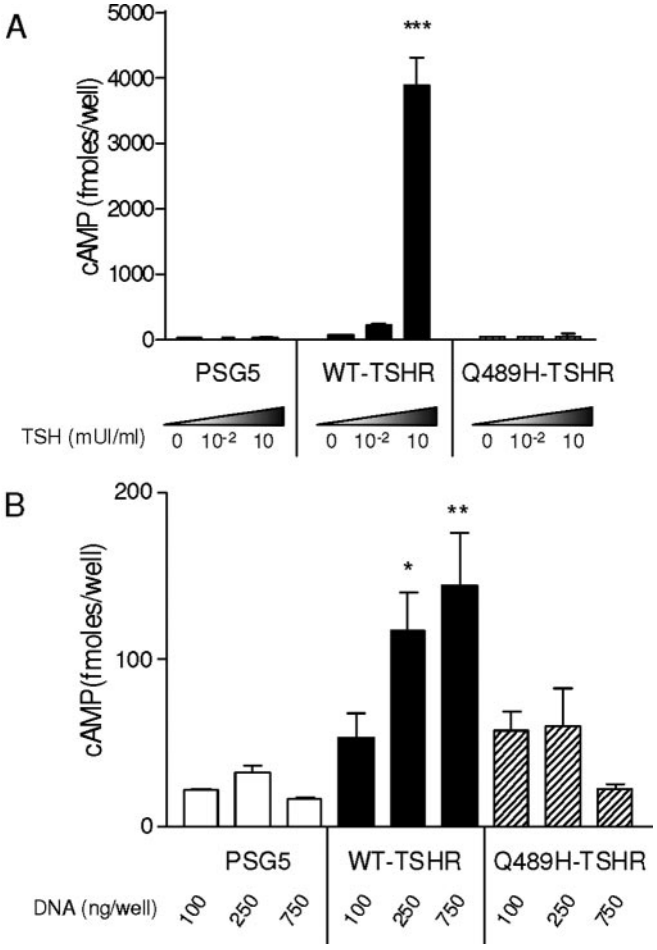

FIG. 2. Q489H substitution impedes adenylate cyclase stimulation by TSHR. HEK293 cells were transiently transfected with the empty expression vector (*white bars*), the vector encoding the wild-type (*black bars*), or the Q489H TSHR (*hatched bars*). A, Stimulation of cAMP accumulation by various concentrations of TSH; B, basal intracellular cAMP levels with increasing amount of transfected DNA (100, 250, or 750 ng/well). Data presented are representative of two independent experiments, each performed in triplicate. *, Significant difference with cells transfected with pSG5 with $P < 0.05$; **, $P < 0.02$.

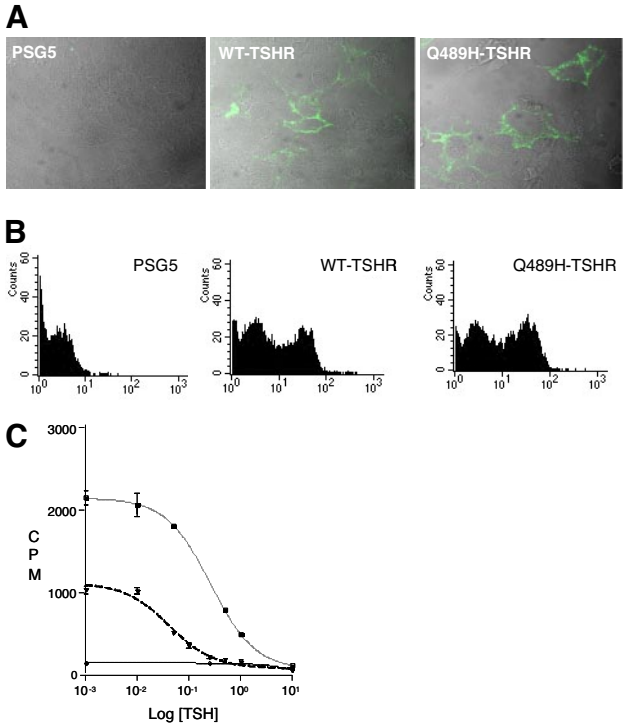

FIG. 3. The Q489H-TSHR is expressed at the cell surface and binds TSH with high affinity. A and B, TSHR expression in nonpermeabilized HEK293 cells evaluated by fluorescent confocal microscopy using the T5–34 antibody (A) and flow immunocytometry (FACS) using the T5–317 antibody (B): TSHR expression at the cell surface in untransfected cells or after transient transfection with empty vector PSG5 (*left*), with the WT-TSHR (*middle*), or with the Q489H mutated TSHR (*right*). Fluorescence intensity in FACS analysis is expressed in arbitrary units, as a function of cell number plotted on a logarithmic scale. C, TSH binding to WT-TSHR and Q489H-TSHR. Cells were transiently transfected with the empty expression vector (◆) or with vectors encoding the wild-type receptor (■) or the Q489H receptor (▼). The cells were incubated with [^{125} I]TSH and increasing concentrations of unlabeled hormone. Values are representative of two independent experiments, each performed in triplicate and are plotted with the SD. CPM, count per minute.

that the Q489H-TSHR had a lower maximum number of TSH-binding sites at the cell surface than the wild-type receptor. Both receptors showed similar affinity for TSH (Fig. 3C).

Evidence that the Q489H-TSHR expressed at the cell surface does not undergo normal posttranslational maturation

The discrepancy in cell-surface expression between FACS and TSH binding may be ascribable to a conformational change of the ECD resulting from Q489H substitution that may enhance epitope recognition by the T5–317 monoclonal antibody. To compare the molecular forms produced from the mutant and wild-type genes, Western blot analysis was carried out with antibodies directed against the TSHR α-subunit or the TSHR β-subunits of the TSHR on Triton-solubilized membrane extracts from transfected HEK293 cells. Four bands were found from the cells expressing WT-TSHR: a high-molecular-weight band around 200 kDa, a band at 95 kDa with the lowest intensity recognized by both antibodies, the TSHR α-subunit at 56 kDa recognized by the T5–317 antibody (Fig. 4A, *left*), and the TSHR β-subunit at 42 kDa recognized by the T3-365 antibody (Fig. 4A, *right*). In contrast, only the high-molecular-weight form and the 95-kDa band were found on immunoblots of membrane extracts from Q489H-TSHR-transfected cells; neither the α- nor the β-subunit were detected in Q489H-TSHR-transfected cell extracts. Shorter exposure time showed a higher intensity for the high Q489H-TSHR molecular form when compared with WT-TSHR (data not shown).

The Q489H-TSHR produced by transfected HEK293 cells seems to be exclusively composed of the 95-kDa form, suggesting that it did not undergo normal maturation. To evaluate possible posttranslational differences in the glycosylation status between both receptors, PNGase F and EndoH treatments were performed on Triton-soluble extracts from WT-TSHR or Q489H-TSHR transfected cells. PNGase F incubation with WT-TSHR led to a decrease of the molecular weight of the TSHR α-subunit from 56 to 32 kDa, confirming that the TSHR α-subunit bears complex sugar (Fig. 4B). For both receptors, EndoH and PNGase F led to similar decrease of the 95-kDa band to 80 kDa. The 95-kDa band thus corresponds to a high-mannose form (Fig. 4B). It should be noted that a small shift from 56 to 52 kDa has been observed on the TSHR α-subunit from WT-TSHR after

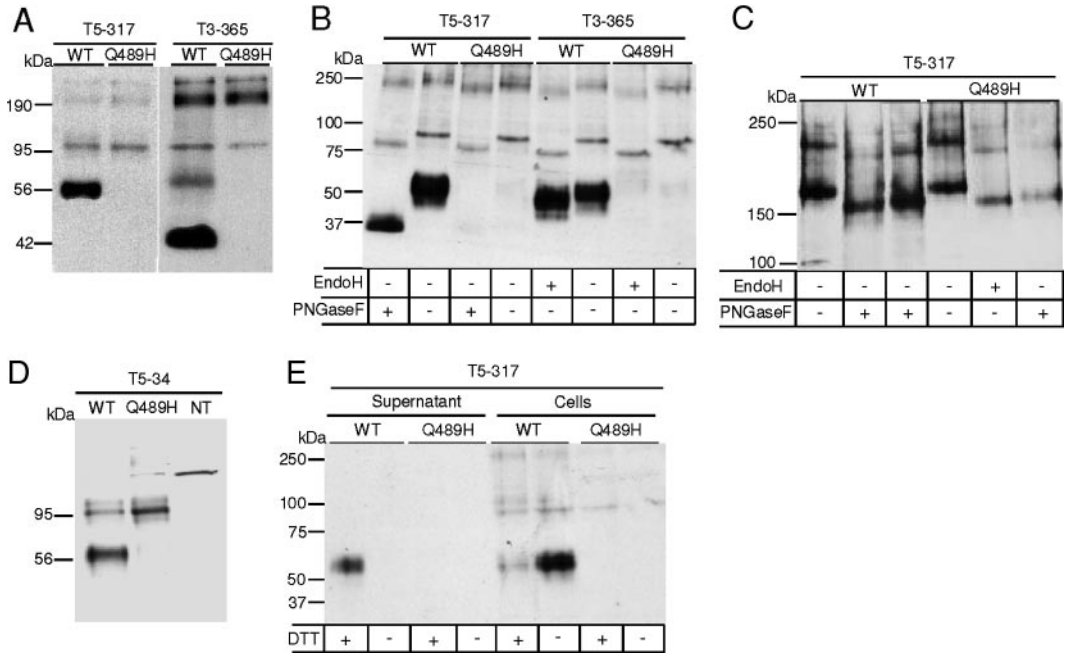

FIG. 4. Molecular characterization of the Q489H-TSHR produced and found at the cell surface. A, Crude membrane preparations of transfected cells were subjected to 8% SDS-PAGE under reducing conditions. After transfer to a membrane, proteins were probed with antibody T5–317 directed against the ECD (*left*) and antibody T3-356 against the TMD (*right*). Four bands at 42, 56, 95, and 190 kDa were observed for the WT-TSHR, whereas only two bands at 95 and 190 kDa were observed for the Q489H-TSHR. B, Triton-solubilized WT-TSHR and Q489H-TSHR from membrane extracts were incubated with EndoH or PNGase F for 1 h at 37 C. Immunoblot was performed with T5–317 and T3-365 antibodies after electrophoresis in an 8% polyacrylamide gel. C, Western blot was performed using T5–317 as in A except that electrophoresis was performed in a 5% polyacrylamide gel. D, Cell surface proteins have been biotinylated as described in *Materials and Methods*. Western blot was performed with T5–34 antibody on NeutrAvidin beads-purified biotinylated proteins. The blot is representative of three independent experiments. E, WT-TSHR or Q489H-TSHR transiently expressing cells were detached from plates and incubated with or without 150 mM DTT for 30 min at room temperature to shed the ECD. Solubilized cell membranes and concentrated supernatants were analysis by Western blot using the T5–317 antibody. Molecular size standards (in kilodaltons) are indicated on the *left* of each blot.

EndoH treatment, indicating the persistence of high-mannose sugars in the cleaved mature TSHR. A decrease of the high-molecular-weight band was observed for both receptors, but the gel resolution did not allow us to determine the exact sizes; therefore, we performed another migration of the same samples in a 5% polyacrylamide gel followed by the same immunoblot conditions. The size of the higher-molecular-weight band was estimated around 190 kDa and an identical change from 190 to 160 kDa was observed after EndoH and PNGase F treatments for both WT-TSHR and Q489H-TSHR (Fig. 4C), showing that this high-molecular-weight form is composed of high-mannose receptor. The 160-kDa molecular size is concordant with the expected weight of immature uncleaved TSHR dimers.

To confirm that TSH binding sites on cell membrane are composed of immature high-mannose uncleaved receptors, we performed cell surface biotinylation followed by a Western blot on streptavidin-purified cell membrane extract. In WT-TSHR cell extracts, two bands at 95 and 56 kDa were found when only one band at 95 kDa was observed in the Q489H cell extracts (Fig. 4D). The intensity of the 95-kDa band is stronger for Q489H-TSHR than for WT-TSHR, suggesting a higher amount of immature Q489H-TSHR expressed at the cell surface compared with the wild-type receptor.

The absence of bands with a size corresponding to the TSHR α-subunit and TSHR β-subunit in Western blots from Q489H-expressing cells suggested that the mutated receptor able to bind TSH is composed of uncleaved immature receptor. Nevertheless, it was recently reported that the TSHR may also reside into Triton-insoluble membrane microdomains (30, 31). To test the hypothesis that TSH binding on Q489H-TSHR could result from cleaved receptor present into such Triton-insoluble microdomains, we incubated HEK293 cells expressing WT-TSHR or Q489H-TSHR with DTT to induce TSHR α-subunit chemical shedding. After DTT incubation, one band at 56 kDa was found in the supernatant of WT-TSHR-expressing cells, whereas no band was observed in the supernatant of cells expressing Q489H-TSHR (Fig. 4E, *left*). Western blot analysis of solubilized membrane extracts from DTT-treated cells expressing WT-TSHR showed a decrease of the 56-kDa band intensity, whereas, as expected, a strong band at 56 kDa was revealed from membrane extracts of untreated cells (Fig. 4E, *right*).

WT-TSHR and Q489H-TSHR follow similar intracellular routes to reach cell surface

We had evidence that HEK293 cell surface expression of mutant Q489H-TSHR is restricted to uncleaved immature receptor. This unexpected observation could be related to a deviation of the intracellular trafficking of the mutant compared with the wild-type receptor, and therefore, Q489H substitution may direct mutant TSHR molecules toward a Golgi-independent intracellular route to reach the cell surface. To test this hypothesis, a fluorescent microscopy analysis with ER or Golgi apparatus markers was carried out in WT-TSHR and Q489H-TSHR-transfected cells. A dual intracellular labeling performed 24 h after

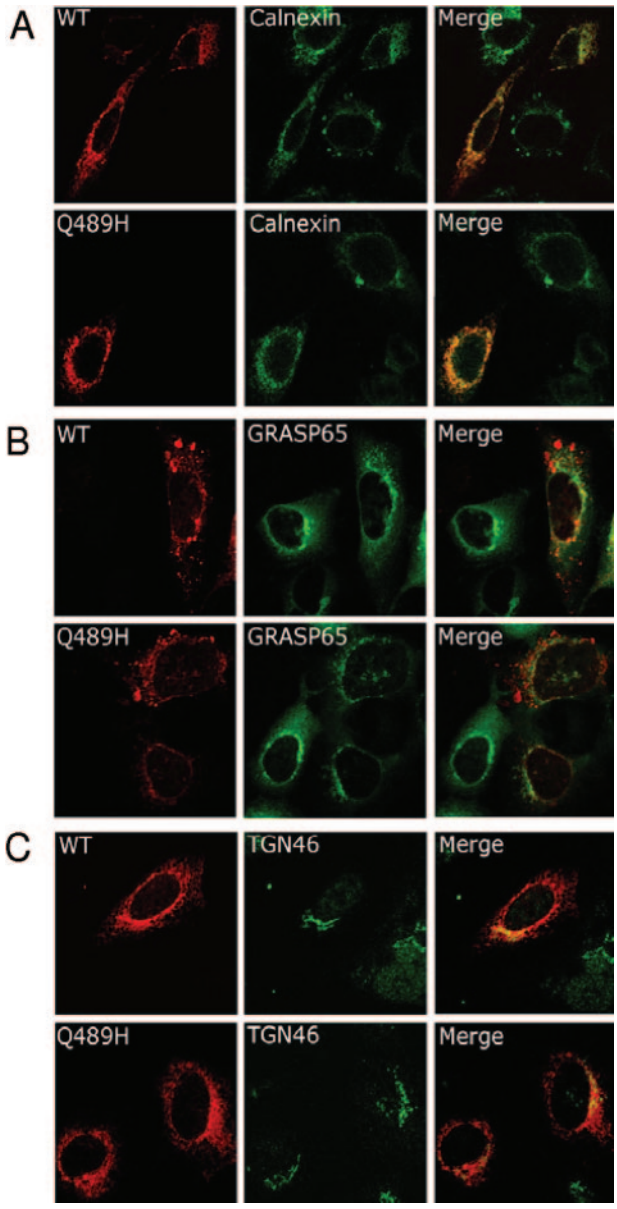

FIG. 5. The intracellular trafficking of WT-TSHR and Q489H-TSHR are similar in HEK293 transfected cells. A and C, HEK293 cell transiently transfected with a plasmid expressing WT-TSHR or Q489H-TSHR were fixed in paraformaldehyde, permeabilized with Triton or with saponin, incubated with T3-365 against TSHR and with an antibody against either calnexin (A) or TGN46 (C). B, HEK293 cells were transiently cotransfected with a plasmid encoding for GRASP65-GFP and either WT-TSHR or Q489H-TSHR, fixed in paraformaldehyde, permeabilized with Triton, and then incubated with the T3-365 antibody.

transfection with antibodies directed against an ER marker, calnexin, and TSHR showed colocalization with both WT-TSHR and Q489H-TSHR within the perinuclear region (Fig. 5A). GRASP65 was recently described as being involved in the exit of proteins from ER and in the formation of the *cis*-Golgi (32). A double transfection of HEK293 cells with a GRASP65-GFP and TSHR expressing plasmids showed that GRASP65 is colocalized with WT-TSHR or Q489H-TSHR within the perinuclear region (Fig. 5B). Apart from the perinuclear region, GRASP65 and WT-TSHR or Q489H-TSHR are localized within different vesicles (Fig. 5B). Colabeling with T3-365 antibody and an antibody against TGN46, a *trans*-Golgi-specific protein revealed that *trans*-Golgi saccules showed a strong TSHR immunoreactivity, suggesting that both wild-type and mutant receptors traffic through the *trans*-Golgi network to reach cell surface (Fig. 5C).

Discussion

Few cases of congenital hypothyroidism due to thyroid dysgenesis have been ascribed to specific genetic abnormalities. Although TSHR defect probably is a rare cause of autosomal recessive hypothyroidism, TSHR mutations are the most common identified cause of thyroid dysgenesis. In this study, we describe a new loss-of-function TSHR mutation associated with familial thyroid dysgenesis. Direct TSHR gene sequencing revealed a unique homozygous Q489H mutation in two siblings with hypothyroidism. The mother and sister were heterozygous for the same mutation and presented a moderate TSH resistance as has been previously described for other TSHR mutations (13, 20, 33, 34).

The 1467C→G mutation leads to a highly conserved polar amino acid substitution (glutamine) with a positively charged amino acid (histidine) in the ECL1 of the TMD. Functional properties of the Q489H mutant receptor were studied after transient transfection in HEK293 cells. The Q489H-TSHR is expressed at the cell surface as an immature mannose-rich receptor, proving that in HEK293 cells, *N*-glycosylation with complex sugars of TSHR is not required for cell membrane addressing. Mannose-rich proteins usually are considered as ER proteins. However, few works have already shown or suggested cell surface expression of such mannose-rich glycoproteins, which was described as a consequence of protein overexpression leading to saturation of the glycosylation machinery within Golgi apparatus (35). The absence of any mature TSHR molecules in Q489H-TSHR-transfected cells indicates that this explanation is not applied to our results.

Several mutations of GPCR impeding cell surface expression have been described. It is considered that these mutant receptors are misfolded and do not pass quality control within the ER, undergoing degradation without exiting the ER. An accumulation of mutant proteins within post-ER compartments such as ER/Golgi intermediate compartment has been reported for the V2 vasopressin receptor; however, these mutant receptors are then retranslocated toward the ER without being expressed at the cell surface (24). Our results showing that mannose-rich TSHR may reach the cell surface are in agreement with the very poor expression of TSHR on the cell surface of transfected CHO-Lec1 cells deficient for *N*-acetylglucosaminyl-transferase I activity (11). It is interesting to note that TSHR expressed in this cell line was reported to be cleaved as the wild-type receptor (11).

Western blot analysis has shown that Q489H substitution did not interfere with the homodimerization of immature forms, which primarily involves the heptical domains and seems to be necessary to pass quality-control checkpoints (36–38). Q489H mutant receptor did not impede cell surface expression of wild-type receptor when both receptors are coexpressed within HEK293 cells (data not shown), which is different from other GPCR (39). The ER quality control thus seems more permissive

for TSHR than for other GPCR. This capacity of immature TSHR to exit ER and traffic throughout the Golgi apparatus to reach the cell surface may be ascribed to the presence of a signal peptide at the N-terminal end, which facilitates the entry into the secretory pathways of folded TSHR when compared with GPCR without evident signal peptide, which use the first transmembrane domain as an anchor signal (40). Q489H is located within the ECL1; the ECD of the Q489H-TSHR has thus probably penetrated within the ER and already interacted with proteins involved in ER quality control such as calnexin (40) when residue 489 is added to the nascent polypeptide. This interaction might be sufficient to engage the Q489H-TSHR toward the cell surface route.

Western blot analysis shows that the Q489H substitution does not impede initial mannose transfer on ECD. Because Q489H-TSHR traffics throughout the Golgi apparatus to reach the cell surface, it is highly probable that the persistent high-mannose glycosylation on Q489H-TSHR expressed at the cell surface is due to a defect of *N*-acetylglucosamine, galactose, or sialic acid residue addition on mannose residues.

It is usually considered that the proteolytic cleavage of the ECD of TSHR occurs at the cell surface. We suppose that the absence of proteolytic cleavage of Q489H-TSHR ECD is not due to an impaired glycosylation process because TSHR expressed in CHO-Lec1 cells, which restrict protein synthesis to mannose-rich protein, is cleaved into two subunits (11). The ECD misfolding induced by Q489H substitution probably perturbs its interaction with the ECD cleaving enzyme.

The 470-498 region is highly conserved within all members of the GPHR subfamily, and tight molecular and functional interactions occur between ECL and residues of the ECD (29, 41). Functional characterizations of other point mutations within the ECL1 of GPHR have highlighted its importance in their structure and activity. T477I mutant receptor shows reduced affinity for TSH and complete signal transduction abolition (42). Aspartate residue located at the junction between the TMD and the ECL1 of the TSHR (D474) or the LH/CGR (D397) is important for signal transduction and cell-surface expression but has no effect on binding affinity (43, 44). Despite their reduced cell-surface expression, activating mutations within the same region (I486F and I486M) lead to constitutive activation of cAMP and phospholipase C transduction pathways, (45). Our results confirm the fundamental role of ECL1 in TSHR activation and folding, although the consequences in activation, binding affinity, or membrane addressing are specific to each of these mutations.

The TSHR domain corresponding to the leucine-rich repeats region devoted to TSH binding and the TSHR β-subunit involved in signal transduction are linked by a hinge region. Hinge structure alteration by point mutations disturbs TSH binding or signal transduction, suggesting functional and therefore tight molecular interactions between hinge region and ECL (46). Hence, TSHR structure must be considered as a whole. One point mutation within ECL1 can therefore alter the overall conformation of the ECD, probably through modifications of the molecular interactions between the hinge region and ECL. Although this alteration has a limited impact on the ability of Q486H-TSHR to constitutively activate adenylate cyclase in HEK293 cells expressing low levels of mutated receptors, TSH binding failed to activate the adenylate cyclase pathway. The impact of the Q489H substitution on TSH-induced phospholipase C activation must be studied to affirm that this substitution within ECL1 prevents TSHR from achieving a full-agonist conformation upon TSH binding. The functional characterization of Q489H-TSHR confirms that the conformation leading constitutive activation of TSHR is different from ligand-dependent active conformation.

In most TSHR loss-of-function mutations previously described, as well as in this report, patients with hypothyroidism had reduced or even no recognized thyroid tissue, usually accompanied by elevated or normal thyroglobulin (Tg) levels (12, 14, 15, 18–22). It would be interesting to study the implication of the surface expression and the stability of an immature TSHR into the cell membrane structure to evaluate its role in the thyroid follicular cell polarization or, as previously suggested, in the leakage phenomenon leading to disproportionate Tg serum levels (12).

In conclusion, this study shows that Q489H-TSHR is expressed as an immature protein at the cell surface and that it is unable to activate adenylate cyclase under TSH stimulation even though it binds TSH with normal affinity. We also bring evidence that a substitution within the ECL1 induces ECD misfolding that interferes with normal interaction of this domain with enzymes involved in the posttranslational maturation of TSHR.

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